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Note

Rapid method of analyzing phenolic compounds in *Pinus elliotti* using high-performance liquid chromatography

B. A. CHARPENTIER and JOE R. COWLES*
Department of Biology, University of Houston, Houston, TX 77004 (U.S.A.)
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Most published analyses of *Pinus* constituents have involved separations by paper chromatography (PC)[1–4] or thin-layer chromatography (TLC)[4–6]; in one instance, high-performance liquid chromatography (HPLC) has been used to investigate extracts from needles of *Pinus jeffreyi*[7]. In all these investigations, the tissue extracted has been from mature trees (needles[5,7], bark[8], pollen[4], heart wood or sapwood[1,2,3,6]). The present work was undertaken to develop a rapid and reliable method for analyzing the phenolic content in hypocotyl sections of young (5–21 days) rapidly developing pine seedlings.

HPLC has been shown to be an effective method for separating standard solutions of phenolic compounds[9,10], and it has been successfully applied to several plant systems[11–14]. This paper reports a method in which HPLC is used to separate and quantitate benzoic acids, cinnamic acids and flavonoids extracted from pine seedlings. It is rapid, reproducible, requires no derivatization and can be applied to small quantities of tissue.

MATERIALS AND METHODS

Standards

Standards were obtained from commercial sources. L-Phenylalanine, D-phenylalanine, hydroquinone, scopoletin, kaempferol, quercetin, and caffeic, *trans*-cinnamic, *p*-coumaric, ferulic, gallic, *p*-hydroxybenzoic, phenylacetic, protocatechuic, shikimic, syringic and vanillic acids were obtained from Sigma (St. Louis, MO, U.S.A.). *o*-Hydroxycinnamic acid, sinapic acid, chrysin, flavanone, phenylacetaldehyde and umbelliferone were obtained from Pfaltz and Bauer (Stamford, CT, U.S.A.).

Instrumentation

Separations were carried out on a liquid chromatograph from Laboratory Data Control. The instrument was equipped with a Constametric I Solvent Delivery System, a Spectromonitor III variable-wavelength detector, and an LDC Chromatography Accessory Module. The recorder was a Fisher Recordall Series 5000. The column system consisted of a Whatman (Clifton, NJ, U.S.A.) Co:Pell ODS pre-column and a Waters (Milford, MA, U.S.A.) μBondapak C_{18} (30 cm × 4 mm I.D.) analytical column. The eluate was monitored at 254 nm.

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Solvents

The eluting solvent consisted of water, acetonitrile [HPLC grade (Burdick and Jackson Labs, Muskegon, MI, U.S.A.)] and acetic acid in the following ratios: (85:10:5), (86:10:4), (87:10:3), (88:10:2), (89:10:1), (78:20:2), (68:30:2), (95:5:0), (90:5:5), (95:0:5) and (70:30:0). All solvents were filtered through 2-μm filters (Millipore Type FG) and degassed before use.

Extraction of pine tissue

The extraction and hydrolysis of pine tissues (*Pinus elliotti* Engelm.) was a modification of published methods[15]. Tissues were extracted with 2 *N* hydrochloric acid (approximately 25 ml per gram of fresh tissue) for 45 min at 100°C. The acid solution was allowed to cool to room temperature and then extracted twice with equal volumes of ethyl acetate. The combined ethyl acetate fractions were evaporated to dryness, and the phenolic residue was taken up in a measured amount of methanol for chromatography.

RESULTS AND DISCUSSION

The retention times for the 22 phenolic standards run in water–acetonitrile–acetic acid (88:10:2) are shown in Table I. A typical chromatogram of ten benzoic and cinnamic acid standards is shown in Fig. 1. Omission of acetic acid from the solvent

TABLE I
RETENTION TIMES FOR PHENOLIC STANDARDS
The reversed-phase column was eluted with water–acetonitrile–acetic acid (88:10:2) at a flow-rate of 2 ml/min.

Compound	*Retention time*
Gallic acid	2 min 23 sec
Hydroquinone	2 min 28 sec
D-Phenylalanine	3 min
L-Phenylalanine	3 min
Protocatechuic acid	3 min 20 sec
Shikimic acid	4 min
Gentisic acid	4 min 22 sec
p-Hydroxybenzoic acid	5 min
Caffeic acid	6 min 40 sec
Vanillic acid	6 min 50 sec
Syringic acid	7 min 40 sec
p-Coumaric acid	12 min
Umbelliferone	12 min
Phenylacetic acid	12 min 30 sec
Phenylacetaldehyde	13 min 8 sec
cis-Ferulic acid	14 min
Scopoletin	15 min
trans-Ferulic acid	16 min
Sinapic acid	20 min
o-Hydroxycinnamic acid	24 min 50 sec
Quercetin	155 min
Kaempferol	>160 min

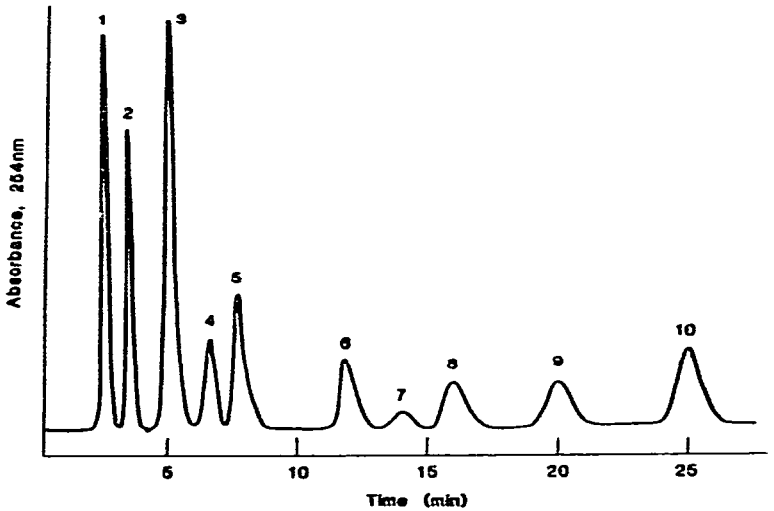

Fig. 1. Separation of benzoic and cinnamic acid standards. The solvent was water–acetonitrile–acetic acid (88:10:2). Peaks: 1 = gallic acid; 2 = protocatechuic acid; 3 = *p*-hydroxybenzoic acid; 4 = caffeic acid; 5 = syringic acid; 6 = *p*-coumaric acid; 7 = *cis*-ferulic acid; 8 = *trans*-ferulic acid; 9 = sinapic acid; 10 = *o*-hydroxycinnamic acid.

generally resulted in broader peaks and longer elution times. In some instances, however, the omission of acetic acid improved the separation. *p*-Coumaric acid and umbelliferone had identical elution times, and ferulic acid and scopoletin were not completely separated when the standard solvent was used at pH 2.8 or less. In water–acetonitrile (90:10), each pair of compounds could be separated. An increase in the acetic acid content of the solvents in general shortened the elution times and, at the highest concentration (5%), caused several of the compounds to merge. Increase in the amount of acetonitrile in the solvent shortened the elution times of the standards. A water–acetonitrile–acetic acid ratio of 68:30:2 caused the benzoic and cinnamic acids to merge, but was useful for the rapid separation of flavonoids (Fig. 2).

The effects of substitution in the aromatic ring on retention times were apparent in the reversed-phase chromatographic system. The addition of a hydroxyl group shortened elution times: gallic acid was the most rapidly eluted of all the compounds run. With the flavonoids, the addition of one hydroxyl group to the B-ring alone resulted in a substantially shorter elution time: quercetin (5 min) compared with kaempferol (8 min) at the solvent ratio of 68:30:2 (Fig. 2). In contrast, the addition of methoxyl groups to the ring increased retention times: *p*-hydroxybenzoic (5 min), vanillic acid (7 min) and syringic acid (8 min); *p*-coumaric acid (12 min), ferulic acid (16 min) and sinapic acid (20 min).

Extracts of 10-day-old pine seedlings were chromatographed in water–acetonitrile–acetic acid (88:10:2) to separate benzoic and cinnamic acids (Fig. 3) and in (68:30:2) to separate the flavonoids (Fig. 4). Peaks 2, 3 and 7 in Fig. 3 have been positively identified as protocatechuic acid, *p*-hydroxybenzoic acid and ferulic acid, respectively, and the two peaks in Fig. 4 as quercetin and kaempferol. The compounds also have been quantitated by using known amounts of standards (unpublished data).

This investigation has shown HPLC to be a powerful tool for the separation of a range of phenolic compounds and to be effective in the analysis of extracts from

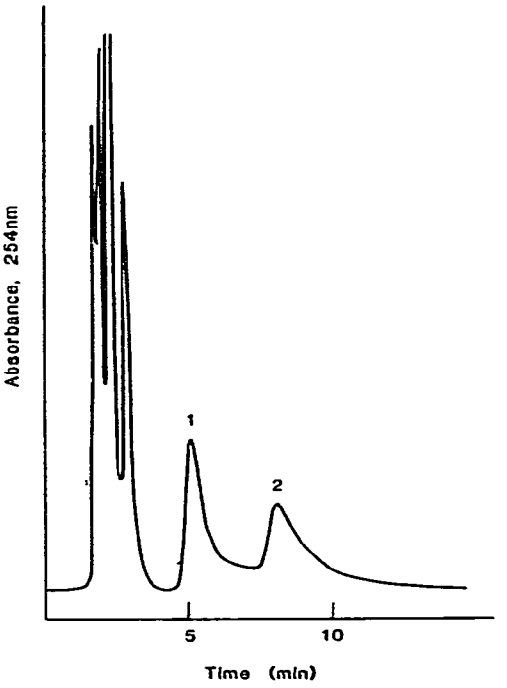

Fig. 2. Separation of two flavonoid standards. The solvent was water–acetonitrile–acetic acid (68:30:2). Peaks: 1 = quercetin; 2 = kaempferol. The mixture also contained the ten standards shown in Fig. 1.

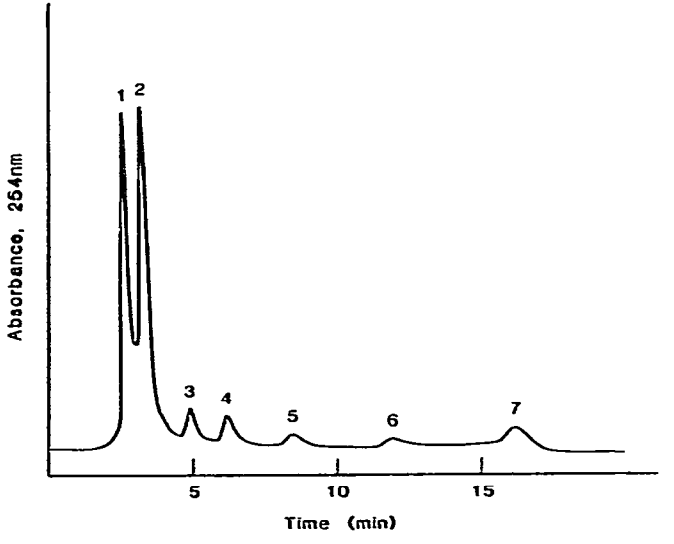

Fig. 3. Chromatographic analysis of an extract from 10-day-old pine seedlings. The solvent was water–acetonitrile–acetic acid (88:10:2). Peaks: 2 = protocatechuic acid; 3 = *p*-hydroxybenzoic acid; 7 = ferulic acid.

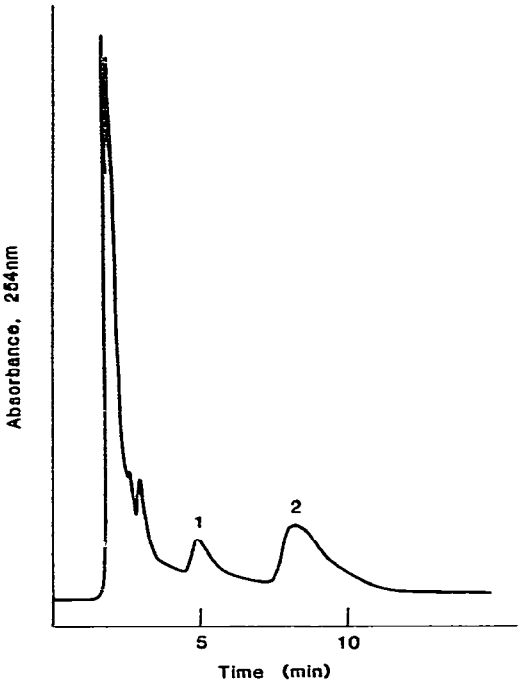

Fig. 4. Chromatographic analysis of an extract from 10-day-old pine seedlings. The solvent was water–acetonitrile–acetic acid (68:30:2). Peaks: 1 = quercetin; 2 = kaempferol.

means of observing and quantitating changes in the soluble phenolic content of small tissue samples and is particularly useful in studying changes in rapidly developing tissues.

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